JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 2000, p. 4649–4652 Vol. 38, No. 12
0095-1137/00/$04.00+0

Characterization of Non-Type B *Haemophilus influenzae* Strains Isolated from Patients with Invasive Disease

MARINA CERQUETTI,[1]* MARTA LUISA CIOFI DEGLI ATTI,[2] GIOVANNA RENNA,[1] ALBERTO EUGENIO TOZZI,[2] MARIA LAURA GARLASCHI,[3] PAOLA MASTRANTONIO,[1] AND THE HI STUDY GROUP†

Laboratory of Bacteriology and Medical Mycology[1] and Laboratory of Epidemiology and Biostatistics,[2] Istituto Superiore di Sanità, 00161 Rome, and Azienda Ospedaliera Istituti Clinici di Perfezionamento, 20122 Milan,[3] Italy

Received 5 May 2000/Returned for modification 6 July 2000/Accepted 10 September 2000

Forty-one non-type b *Haemophilus influenzae* isolates from cases of invasive disease were characterized. By PCR capsular genotyping, 33 nonencapsulated strains, 4 type f isolates, and 4 b$^-$ strains were identified. By pulsed-field gel electrophoresis, the nonencapsulated isolates exhibited great genetic heterogenicity, whereas the type f and the b$^-$ strains seemed to have a clonal spread. Occurrence of the *hifA* gene was found by PCR in 18% of the nonencapsulated, 50% of the b$^-$, and all of the type f strains. Hemagglutinating fimbriae were generally expressed by nonencapsulated isolates when fimbrial gene *hifA* was present. Two nonencapsulated isolates not susceptible to ampicillin were detected; no strains were positive for β-lactamase production.

Haemophilus influenzae is responsible for a variety of localized respiratory tract infections and invasive diseases (e.g., meningitis, septicemia, epiglottitis, and septic arthritis) (29). Invasive disease is associated with a minority of virulent strains, generally encapsulated *H. influenzae* serotype b (Hib) (35); however, other serotypes or nonencapsulated strains have also been found responsible. With the advent of effective conjugated vaccines against the Hib capsular antigen, serotype b disease has declined in many industrialized countries (1). It has been speculated that, with the decrease of Hib, other serotypes and nonencapsulated strains will become relatively more important (25, 34). Besides, the extensive use of Hib vaccine may produce an increase of invasive *H. influenzae* disease, due to spontaneous capsule-deficient mutants of serotype b (b$^-$ strains), since these strains are not susceptible to antibodies elicited by the vaccine (12). Careful analysis of *H. influenzae* strains isolated from patients with invasive diseases will be increasingly important.

In Italy, the first Hib conjugate vaccine was licensed in February 1995, and vaccination is voluntary. Vaccination coverage is currently low; in 1998, it was an estimated 19.8% for the 1996 birth cohort between 12 and 24 months of age (23). The aim of the present study was to characterize the non-Hib strains isolated from patients with invasive disease in Italy, focusing on the following three major items. First, we investigated the genetic relationship among isolates by pulsed-field gel electrophoresis (PFGE). Second, we assessed the presence of hemagglutinating fimbriae by PCR to detect the *hifA* gene, encoding the major subunit of the fimbriae, and by hemagglutination assay to demonstrate its phenotypic expression. Finally, we tested the strains for β-lactamase production and intrinsic ampicillin resistance.

Bacterial strains. Forty-one non-Hib isolates recovered from patients with invasive disease in Italy, between April 1994 and December 1998, were analyzed. Thirty-two strains were obtained from cases detected through the Active Surveillance Program on *H. influenzae* Invasive Disease; 9 other strains were isolated from cases reported to the National Surveillance System for Bacterial Meningitis (4). One Hib isolate belonging to the clone endemically present in Italy (27) was also included in the study.

Capsular genotyping of *H. influenzae* isolates and characteristics of cases. For PCR capsular genotyping, two separate amplifications of the target DNA were carried out. In a first round of PCR, primers omp1 and omp2 (11) directed to the *ompP2* gene were used to confirm the *H. influenzae* species, while primers HI-1 and HI-2 (7) directed to the *bexA* region proved capsulation. In a second round of PCR, primers directed to each capsule type-specific region (7) were used. Primers were supplied by M-Medical, Florence, Italy. Preparations of total DNA and amplification reactions were performed as previously described (7). Samples underwent 25 cycles in a Perkin-Elmer Cetus 9600 instrument with the following parameters: denaturation at 94°C (1 min), annealing at 55°C (1 min), elongation at 72°C (1 min), and finally 8 min of incubation at 72°C. The resulting PCR products were electrophoresed through 1.5% agarose (Roche Diagnostics GmbH, Mannheim, Germany) in Tris-borate-EDTA buffer and visualized by ethidium bromide staining. By this method, 33 nonencapsulated strains, 4 type f isolates, and 4 b$^-$ strains were identified among our isolates. The 33 cases due to nonencapsulated strains were equally distributed among sexes. The median age was 52 years (range, 5 months to 91 years). Bacteremia with other nonidentified foci was the most frequent clinical presentation (41.4%), followed by meningitis (31%). Of the cases,

* Corresponding author. Mailing address: Laboratorio di Batteriologia e Micologia Medica, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy. Phone: 3906 49902343. Fax: 3906 49387112. E-mail: mcerquet@iss.it.

† Members of The Hi Study Working Group: Istituto Superiore di Sanità, Laboratorio di Epidemiologia e Biostatistica, Patrizia Carbonari; Associazione Microbiologi Clinici Italiani (AMCLI), Pierluigi Nicoletti and Antonio Goglio; Regione Piemonte, Angela Ruggenini Moiraghi, Stefania Orecchia, Annalisa Castella, and Carla Zotti; Regione Lombardia, Alessandro Lizioli and Salvatore Pisani; Provincia Autonoma di Trento, Valter Carraro, Iole Caola, and Anna Calì; Regione Veneto, Giovanni Gallo; Regione Liguria, Pietro Crovari, Cristina Giordano, Pietro Tixi, and Marina Lemmi; Regione Toscana, Paolo Bonanni, Alessia Tomei, Patrizia Pecile, Emanuela Balocchini, and Licia Pecori; Regione Campania, Francesco Santonastasi, Loredana Cafaro, and Vittorio Pagano; Regione Puglia, Salvatore Barbuti and Maria Chironna.

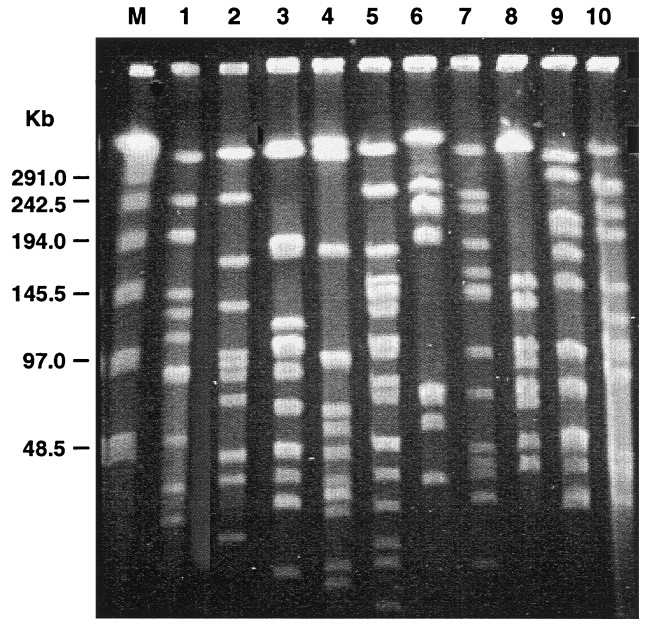

FIG. 1. Examples of PFGE patterns of chromosomal DNAs extracted from nonencapsulated *H. influenzae* isolates. Chromosomal DNA was digested with the *Sma*I restriction endonuclease. Lanes: 1, Hib strain belonging to the clone endemically present in Italy; 2 through 10, nonencapsulated *H. influenzae* strains; M, lamba ladder pulsed-field gel marker. This figure shows nine different patterns among nonencapsulated *H. influenzae* isolates; all of these isolates were unrelated to the Hib strain.

31% presented underlying pathologies, such as cancer. The case fatality rate was 14.3%. All but one of the invasive diseases due to *H. influenzae* type f strains occurred in adult males. On the other hand, three out of the four invasive diseases caused by b^- strains were observed in children under 6 years of age. Meningitis was the most frequent diagnosis in both type f and b^- invasive infections.

PFGE. PFGE was performed by following the procedures previously described (27). All isolates were analyzed using the restriction enzyme *Sma*I (Roche Diagnostics GmbH). Representative PFGE patterns obtained for some nonencapsulated strains are shown in Fig. 1. Thirty different patterns were found among the 33 nonencapsulated isolates. Twenty-seven (90%) were found for single isolates, and each of the other three (10%) was found for two isolates. According to the interpreting criteria reported by Tenover et al. (28), 19 strains showing an individual pattern were considered totally unrelated to each other, whereas 8 showed some degree of relatedness. In particular, six strains isolated from patients from different towns in Lombardia, between October 1997 and January 1998, represented a group of genetically related isolates (data not shown). All of the nonencapsulated strains studied were unrelated to the invasive Hib strain circulating in Italy (Fig. 1). A close genetic relationship was observed among the four *H. influenzae* type f strains. Two of them shared the same restriction pattern (hereafter termed pattern A), while the other two showed profiles which can be considered, respectively, closely and possibly related to pattern A (Fig. 2). Likewise (and hereafter designated pattern B), a similar close genetic relationship was found among the four b^- strains (Fig. 2). The *H. influenzae* type f strains tested can be considered unrelated to the Hib strain, while the b^- strains analyzed were always related to Hib.

Fimbrial expression and detection of the *hifA* gene by PCR. Detection of hemagglutinating fimbriae was carried out by a semiquantitative assay for the ability to agglutinate human erythrocytes as described elsewhere (19). The specificity of hemagglutination for fimbriae was assessed by inhibition with the sialylated ganglioside GM1 (Sigma, St. Louis, Mo.) at a final concentration of 100 μg/ml (31). Five nonencapsulated isolates were positive in the hemagglutination assay, showing titers ranging from 1:8 to 1:16. The presence of the *hifA* gene, which encodes the major subunit of the hemagglutinating fimbriae of *H. influenzae*, was determined by PCR, using primers fgHifA1 and fgHifA2 as described by F. Geluk et al. (9). Primers were supplied by M-Medical. Amplification reactions were performed under the conditions previously described (9). Hib strains 770235 and 760705 (kindly provided by P. van Ulsen, RIVM, Bilthoven, The Netherlands) were used as positive and negative controls, respectively. The presence of the *hifA* gene was revealed by the generation of an 800-bp product in all five of the hemagglutinating isolates and in another seven strains which did not express fimbriae (Fig. 3). Those hemagglutinating negative isolates containing an *hifA* gene included one nonencapsulated, two b^-, and all four type f strains.

Ampicillin susceptibility testing. The minimum inhibitory concentrations (MICs) for ampicillin were determined by E-test (AB Biodisk, Solna, Sweden) using Haemophilus Test Medium agar plates incubated at 37°C for 20 h in a humid atmosphere enriched with 5% CO_2. Reference Hib strain ATCC 10211 was used as the control. The interpretative breakpoints used were based on NCCLS criteria (18). Production of

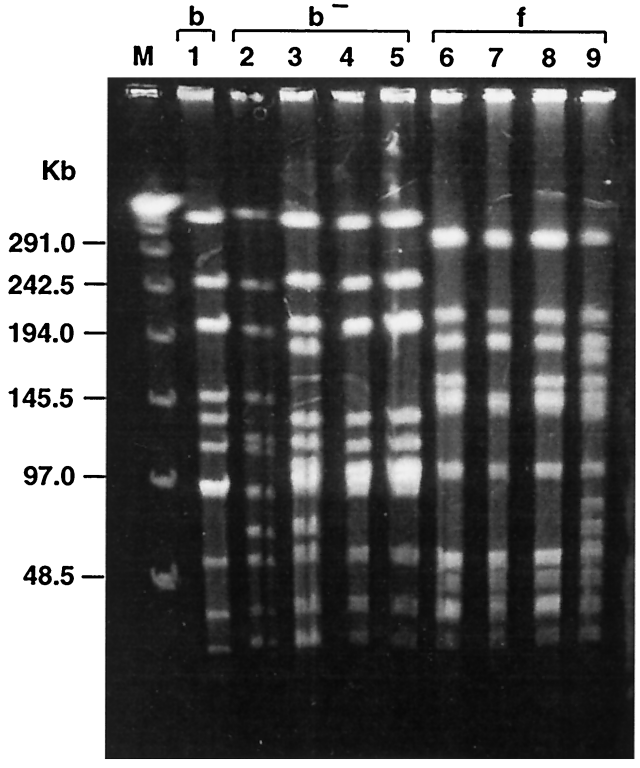

FIG. 2. PFGE patterns of *Sma*I-digested chromosomal DNAs of *H. influenzae* isolates. Capsular type designations are indicated above lanes. M, lamba ladder pulsed-field gel marker. The strains in lanes 4 and 5 showed indistinguishable profiles (pattern B); the strains in lanes 2 and 3 were, respectively, possibly and closely related to pattern B. The strains in lanes 6 and 8 had the same pattern (pattern A), whereas the strains in lanes 7 and 9 were, respectively, closely or possibly related to pattern A.

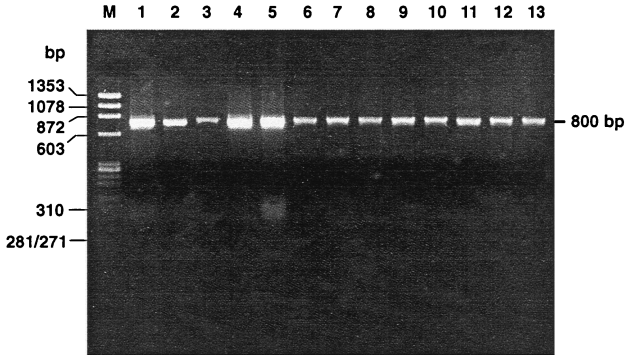

FIG. 3. Agarose gel electrophoresis of PCR products from *H. influenzae* strains amplified with fgHifA1 and fgHifA2 primers. Lanes: 1 to 6, nonencapsulated *H. influenzae* strains; 7 to 10, *H. influenzae* type f strains; 11 and 12, b$^-$ strains; 13, Hib 770235, a positive control; M, DNA molecular mass marker. The generation of an 800-bp product indicated the presence of the *hifA* gene. This figure shows the *hifA*-positive strains recovered in this study.

β-lactamase was detected by the cefinase disk test (Becton-Dickinson, Cockeysville, Md.). The MIC at which 90% of the isolates tested wre inhibited (MIC_{90}) for the 33 nonencapsulated strains was 0.5 μg/ml (range, 0.25 to 8 μg/ml). Thirty-one nonencapsulated strains were susceptible, one was intermediately resistant (MIC, 2 μg/ml), and one was resistant (MIC, 8 μg/ml) to ampicillin. The last strain, isolated in 1998, corresponded to 5% of the nonencapsulated strains isolated that year. All four of the *H. influenzae* type f isolates and the four b$^-$ strains were found to be susceptible to the same MIC, 0.25 μg/ml. No strains were found that produced β-lactamase.

The 41 non-type b *H. influenzae* isolates analyzed in this study were first tested by a PCR genotyping method for the unequivocal assignment of their capsular type. The results obtained indicated that nonencapsulated isolates account for most of the non-type b strains that cause invasive disease in Italy. Moreover, the recovery of b$^-$ strains confirmed the usefulness of PCR capsular genotyping (3). An analysis of cases revealed that invasive disease caused by nonencapsulated and type f strains occurred mostly in adults with diagnosed bacteremia, thus differing from the pattern produced by Hib isolates. On the contrary, the presentation of b$^-$ disease may resemble that of Hib, although the number of b$^-$ strains recovered in the study is too small to be conclusive.

To study the genetic relationships among the different *H. influenzae* isolates, we used PFGE, a well-established methodology, which already has been used to differentiate *H. influenzae* strains (15, 22, 27). The invasive nonencapsulated isolates that we analyzed showed considerable genetic heterogeneity, confirming the earlier described genetic diversity of nonencapsulated *H. influenzae* strains (16, 26, 30). This result extends the data obtained by van Alphen et al. (30) to invasive disease; this study revealed no significant association between specific multilocus genotypes and kinds of disease.

A close genetic relationship was found both among the *H. influenzae* type f strains and among the b$^-$ strains recovered, although the isolates analyzed were few. As expected, the b$^-$ strains tested were genetically related to the invasive Hib strain circulating in Italy. Conversely, the four type f strains were totally unrelated to the same Hib isolate. This finding is in accordance with the data reported by Musser et al. (17) that type c, e, and f strains have no close genetic relationships to strains of other serotypes.

Hemagglutinating fimbriae can be present on both encapsulated and nonencapsulated *H. influenzae* strains, and they have been shown to mediate adherence to human erythrocytes carrying the AnWj antigen and to specific human epithelial cells by binding to the GM1-like receptor (10, 21). A fimbria gene cluster containing the genes *hifA* to *hifE* has been identified (14, 33). It has been reported that the expression of hemagglutinating fimbriae is subject to reversible phase variation (32); during natural infection with Hib, nasopharyngeal isolates are often fimbriated while their isogenic counterparts from blood or cerebrospinal fluid are invariably found to be nonfimbriated (19). Our results seem to indicate that type f and b$^-$ strains behave as Hib strains; that is, systemic isolates do not express fimbriae even if fimbria genes are present. As far as nonencapsulated *H. influenzae* strains are concerned and contrary to results from other studies regarding strains isolated from patients with noninvasive diseases (9, 13), our data demonstrated that nonencapsulated strains isolated from systemic sites generally express fimbriae, if a fimbria gene cluster is present. No association between expression of fimbriae and clinical presentation of the disease was observed. Whether a correlation between the expression of fimbriae and the invasive properties of a subset of nonencapsulated strains may be supposed is still unclear, and much additional work will be required in this area.

In the last decade, *H. influenzae* strains resistant to ampicillin have been isolated with increasing frequency worldwide (5, 8). Plasmid-mediated production of TEM or ROB β-lactamases is the most common mechanism of resistance to ampicillin; however, resistance can also be caused by intrinsic mechanisms involving target modification. Although strains that are β-lactamase negative and ampicillin resistant (BLNAR) are relatively uncommon, their numbers are increasing, mainly among nonencapsulated strains (2, 6, 24, 36).

In the present study, no strains were β-lactamase producers. This result is not surprising since β-lactamase production is more frequent among Hib strains (20), and even there, very few β-lactamase-positive strains have been previously reported in Italy (27). Only one nonencapsulated strain fulfilled the definition of BLNAR; nevertheless this result shows that BLNAR strains may be recovered among invasive nonencapsulated *H. influenzae* strains in Italy. Little is known regarding the clinical relevance of the BLNAR strains; however, our data suggest the need to monitor both the β-lactamase- and non-β-lactamase-mediated resistance mechanisms in nonencapsulated *H. influenzae* isolates.

We are grateful to Fabio D'Ambrosio for technical support in performing PFGE and Tonino Sofia for editorial assistance.

REFERENCES

1. **Adams, W. G., K. A. Dever, S. L. Cochi, B. D. Plikaytis, E. R. Zell, C. V. Broome, and J. D. Wenger.** 1993. Decline of childhood *Haemophilus influenzae* type b (Hib) disease in the Hib vaccine era. JAMA **269:**221–226.
2. **Butt, H. L., A. W. Cripps, and R. L. Clancy.** 1997. In vitro susceptibility patterns of nonserotypable *Haemophilus influenzae* from patients with chronic bronchitis. Pathology **29:**72–75.
3. **Cerquetti, M., G. Renna, and P. Mastrantonio.** 1999. PCR capsular typing of *Haemophilus influenzae* isolates from invasive diseases. Clin. Microbiol. Infect. **5:**195.
4. **Ciofi degli Atti, M. L., A. E. Tozzi, S. Salmaso, M. Cerquetti, and P. Mastrantonio.** 1999. Surveillance of *Haemophilus influenzae* invasive disease in Italy: progress report. Rapporti ISTISAN **99/5:**1–50.
5. **Doern, G. V., and The Alexander Project Collaborative Group.** 1996. Antimicrobial resistance among lower respiratory tract isolates of *Haemophilus influenzae*: results of a 1992–93 Western Europe and USA collaborative surveillance study. J. Antimicrob. Chemother. **38**(Suppl. A)**:**59–69.
6. **Doern, G. V., A. B. Brueggemann, G. Pierce, H. P. Holley, Jr., and A. Rauch.** 1997. Antibiotic resistance among clinical isolates of *Haemophilus influenzae* in the United States in 1994 and 1995 and detection of β-lactamase-positive strains resistant to amoxicillin-clavulanate: results of a national multicenter surveillance study. Antimicrob. Agents Chemother. **41:**292–297.
7. **Falla, T. J., D. W. M. Crook, L. N. Brophy, D. Maskell, J. S. Kroll, and E. R.**

Moxon. 1994. PCR for capsular typing of *Haemophilus influenzae*. J. Clin. Microbiol. **32:**2382–2386.
8. **Felmingham, D., M. J. Robbins, Y. Tesfaslasie, I. Harding, S. Shrimpton, and R. N. Gruneberg.** 1998. Antimicrobial susceptibility of community-acquired lower respiratory tract bacterial pathogens isolated in the UK during the 1995–1996 cold season. J. Antimicrob. Chemother. **41:**411–415.
9. **Geluk, F., P. P. Eijk, S. M. van Ham, H. M. Jansen, and L. van Alphen.** 1998. The fimbria gene cluster of nonencapsulated *Haemophilus influenzae*. Infect. Immun. **66:**406–417.
10. **Gilsdorf, J. R., K. W. McCrea, and C. F. Marrs.** 1997. Role of pili in *Haemophilus influenzae* adherence and colonization. Infect. Immun. **65:**2997–3002.
11. **Hobson, R. P., A. Williams, K. Rawal, T. H. Pennington, and K. J. Forbes.** 1995. Incidence and spread of *Haemophilus influenzae* on an Antarctic base determined using the polymerase chain reaction. Epidemiol. Infect. **114:**93–103.
12. **Hoiseth, S. K., C. J. Connelly, and E. R. Moxon.** 1985. Genetics of spontaneous, high frequency loss of capsule expression in *Haemophilus influenzae*. Infect. Immun. **49:**389–395.
13. **Krasan, G. P., D. Cutter, S. L. Block, and J. W. St. Geme III.** 1999. Adhesin expression in matched nasopharyngeal and middle ear isolates of nontypeable *Haemophilus influenzae* from children with acute otitis media. Infect. Immun. **67:**449–454.
14. **Mhlanga-Mutangadura, T., G. Morlin, A. L. Smith, A. Eisenstark, and M. Golomb.** 1998. Evolution of the major pilus gene cluster of *Haemophilus influenzae*. J. Bacteriol. **180:**4693–4703.
15. **Moor, P. E., P. C. Collignon, and G. L. Gilbert.** 1999. Pulsed-field gel electrophoresis used to investigate genetic diversity of *Haemophilus influenzae* type b isolates in Australia shows differences between Aboriginal and non-Aboriginal isolates. J. Clin. Microbiol. **37:**1524–1531.
16. **Musser, J. M., S. J. Barenkamp, D. M. Granoff, and R. K. Selander.** 1986. Genetic relationships of serologically nontypable and serotype b strains of *Haemophilus influenzae*. Infect. Immun. **52:**183–191.
17. **Musser, J. M., et al.** 1990. Global genetic structure and molecular epidemiology of encapsulated *Haemophilus influenzae*. Rev. Infect. Dis. **12:**75–111.
18. **National Committee for Clinical Laboratory Standards.** 2000. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, vol. 20. Approved standard M7–A5. National Committee for Clinical Laboratory Standards, Wayne, Pa.
19. **Pichichero, M. E., M. Loeb, P. Anderson, and D. H. Smith.** 1982. Do pili play a role in pathogenicity of *Haemophilus influenzae* type b? Lancet **ii:**960–962.
20. **Powell, M., Y. S. Fah, A. Seymour, M. Yuan, and J. D. Williams.** 1992. Antimicrobial resistance in *Haemophilus influenzae* from England and Scotland in 1991. J. Antimicrob. Chemother. **29:**547–554.
21. **Rao, V. K., G. P. Krasan, D. R. Hendrixson, S. Dawid, and J. W. St. Geme III.** 1999. Molecular determinants of the pathogenesis of disease due to non-typeable *Haemophilus influenzae*. FEMS Microbiol. Rev. **23:**99–129.
22. **Saito, M., A. Umeda, and S. Yoshida.** 1999. Subtyping of *Haemophilus influenzae* strains by pulsed-field gel electrophoresis. J. Clin. Microbiol. **37:**2142–2147.
23. **Salmaso, S., M. C. Rota, M. L. Ciofi degli Atti, A. E. Tozzi, P. Kreidl, and The ICONA Study Group.** 1999. Infant immunization coverage in Italy by cluster survey estimates. WHO Bull. **77:**843–851.
24. **Seki, H., Y. Kasahara, K. Ohta, Y. Saikawa, R. Sumita, A. Yachie, S. Fujita, and S. Koizumi.** 1999. Increasing prevalence of ampicillin-resistant, non-beta-lactamase-producing strains of *Haemophilus influenzae* in children in Japan. Chemotherapy **45:**15–21.
25. **Slack, M. P. E., H. J. Azzopardi, R. M. Hargreaves, and M. E. Ramsay.** 1998. Enhanced surveillance of invasive *Haemophilus influenzae* disease in England, 1990 to 1996: impact of conjugate vaccines. Pediatr. Infect. Dis. J. **17:**S204–S207.
26. **Smith-Vaughan, H. C., K. S. Sriprakash, A. J. Leach, J. D. Mathews, and D. J. Kemp.** 1998. Low genetic diversity of *Haemophilus influenzae* type b compared to nonencapsulated *H. influenzae* in a population in which *H. influenzae* is highly endemic. Infect. Immun. **66:**3403–3409.
27. **Tarasi, A., F. D'Ambrosio, G. Perrone, and A. Pantosti.** 1998. Susceptibility and genetic relatedness of invasive *Haemophilus influenzae* type b in Italy. Microb. Drug Resist. **4:**301–306.
28. **Tenover, F. C., R. D. Arbeit, R. V. Goering, P. A. Mickelsen, B. E. Murray, D. H. Persing, and B. Swaminathan.** 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J. Clin. Microbiol. **33:**2233–2239.
29. **Turk, D. C.** 1984. The pathogenicity of *Haemophilus influenzae*. J. Med. Microbiol. **18:**1–16.
30. **van Alphen, L., D. A. Caugant, B. Duim, M. O'Rourke, and L. D. Bowler.** 1997. Differences in genetic diversity of nonencapsulated *Haemophilus influenzae* from various diseases. Microbiology **143:**1423–1431.
31. **van Alphen, L., L. G. van den Broek, L. Blaas, M. van Ham, and J. Dankert.** 1991. Blocking of fimbria-mediated adherence of *Haemophilus influenzae* by sialyl gangliosides. Infect. Immun. **59:**4473–4477.
32. **van Ham, S. M., L. van Alphen, F. R. Mooi, and J. P. van Putten.** 1993. Phase variation of *Haemophilus influenzae* fimbriae: transcriptional control of the two divergent genes through a variable combined promoter region. Cell **73:**1187–1196.
33. **van Ham, S. M., L. van Alphen, F. R. Mooi, and J. P. M. van Putten.** 1994. The fimbrial gene cluster of *Haemophilus influenzae* type b. Mol. Microbiol. **13:**673–684.
34. **Waggoner-Fountain, L. A., J. O. Hendley, E. J. Cody, V. A. Perriello, and L. G. Donowitz.** 1995. The emergence of *Haemophilus influenzae* type e and f as significant pathogens. Clin. Infect. Dis. **21:**1322–1324.
35. **Wenger, J. D., A. W. Hightower, R. R. Facklam, S. Gaventa, and C. V. Broome.** 1990. Bacterial meningitis in the United States, 1986: report of a multistate surveillance study. J. Infect. Dis. **126:**1316–1323.
36. **Williams, J. D., M. Powell, Y. S. Fah, A. Seymour, and M. Yuan.** 1992. In vitro susceptibility of *Haemophilus influenzae* to cefaclor, cefixime, ceftamet and loracarbef. Eur. J. Clin. Microbiol. Infect. Dis. **11:**748–751.